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Phylogenetic analysis of hepatitis C virus isolates from hemodialysis patients

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Phylogenetic analysis of hepatitis C virus isolates from hemodialysis patients. A high prevalence of hepatitis C virus (HCV) infection has been reported in hemodialysis patients. Main risk factors for transmission are previous blood transfusions and possibly nosocomial infections within the dialytic environment. In the present study 224 hemodialysis patients from the same department were tested for the presence of anti-HCV antibodies and HCV-RNA. The presence of anti-HCV in hemodialysis patients was correlated with a history of more than 10 blood transfusions ($P = 0.001$) and with a duration of hemodialysis treatment for more than 10 years ($P = 0.001$). The issue of possible patient-to-patient infection was addressed by sequence analysis of all HCV-RNA positive hemodialysis patients ($N = 14$) together with a control panel of HCV isolates from 56 unrelated non-hemodialysis patients with hepatitis C from the same geographical area. Subsequent phylogenetic analysis of nucleotide sequences obtained from the 5′-noncoding region and the nonstructural NS-5 region of the HCV genome revealed that only two hemodialysis patients were infected by a highly related HCV isolate. The remaining HCV-RNA positive hemodialysis patients including those without previous blood transfusions were all infected by phylogenetically-distant HCV isolates, providing evidence against a nosocomial transmission route. The data of the present study show that molecular epidemiological techniques are important to investigate the issue of nosocomial infection. In our hemodialysis unit patient-to-patient infection appears uncommon and draws attention towards other possible (such as, blood products such as human serum albumin, immunoglobulins) or even yet unrecognized transmission routes.

The main route of hepatitis C virus (HCV) transmission is parenteral, and many HCV-infected individuals are recipients of blood and blood products that in the past had not been screened for anti-HCV. HCV infection is also frequently associated with intravenous drug abuse. Smaller numbers of HCV infections are related to sexual or vertical transmission, organ transplantation, tattoos and piercing, blood suction practices in folk medicine, etc. [1, 2]. For many patients infected with the hepatitis C virus, however, no source of infection can be established [3, 4]. The availability of tests for antibodies to HCV and the use of the polymerase chain reaction (PCR) to detect HCV-RNA has enabled a more definitive characterization of the epidemiology and routes of transmission.

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A high prevalence of antibodies to hepatitis C virus has been reported in hemodialysis patients [5–10]. Before the introduction of recombinant erythropoietin, blood transfusions were a common therapeutic approach to treat anemia in hemodialysis patients. Beside the risk of HCV transmission via transfusion of blood and blood products, previous renal transplantations and/or insufficient infection control procedures in the hemodialysis facilities itself might account for the high prevalence of HCV infection in these patients. Several studies supported the hypothesis for procedure-related transmission of HCV [11–13]. Evidence for HCV transmission within patients of a hemodialysis unit has major impact on hygienic routines, sterilization procedures and the necessity of segregation of HCV-positive patients.

Methods

Patients, blood samples, and serological tests

The study population consisted of 224 HBsAg-negative patients (126 male, 98 female) with a mean age of 56.7 years (range 19 to 79 years) receiving maintenance hemodialysis at our University Hospital-based dialysis unit. Hemodialysis was performed three times weekly, each time for four hours, using cuprophane, polymethilmetacrylate, polysulphone or cellulose acetate membranes. The number of blood transfusion units received, previous kidney transplantations, and the duration of hemodialysis treatment were recorded at the beginning of the study in 1993. Fifty-six consecutive patients with chronic hepatitis C and no evidence of renal disease attending our hepatology out-patient clinic served as unrelated controls. All patients were at least 18 years old and informed consent was obtained. Blood samples from all patients were centrifuged within two hours to achieve optimal conditions for HCV-RNA determination [14]. Serum was prepared under a laminar flow bench and frozen at −80°C. Testing for anti-HCV (2nd generation test), HBsAg, anti-HBc, anti-HIV I and II were performed using commercially available ELISAs or radioimmunoassays. Each patient was tested for the presence of HCV-RNA by RT-PCR.

RT-PCR and sequencing of HCV isolates

A RT-PCR assay was performed essentially as described [15–17]. For extraction of RNA 100 μl aliquots of frozen serum were thawn and RNA was extracted by acid guanidinium thiocyanate-phenol-chloroform extraction [18]. After washing, the RNA pellet

Table 1. Demographic, clinical and biochemical features of patients

	Patients on hemodialysis		Controls
	Anti-HCV negative (N = 208)	Anti-HCV positive (N = 16)	Anti-HCV positive (N = 56)
Age *years*	57.2 ± 13.2	50.1 ± 17.3	47.9 ± 14.8
Sex (male/female)	114/94	12/4	29/27
HCV-RNA	0 (0%)	14 (87.5%)	56 (100%)
HBsAg	0 (0%)	0 (0%)	0 (0%)
Anti-HBc	48 (23.1%)	5 (31.3%)	17 (30.4%)
Anti-HIV	0 (0%)	0 (0%)	0 (0%)
ALT *U/liter*	7.8 ± 7.3	19.6 ± 17.2	84.1 ± 47.7

Table 2. Anti-HCV antibodies in relation to previous blood transfusions in patients on chronic hemodialysis

Number of transfusions *units*	Anti-HCV negative patients (N = 208)	Anti-HCV positive patients (N = 16)	P value
0	81 (38.9%)	3 (18.8%)	NS
1–2	41 (19.7%)	1 (6.2%)	NS
3–10	45 (21.7%)	5 (31.3%)	NS
> 10	41 (19.7%)	7 (43.7%)	0.001

was vacuum dried for 15 minutes and resuspended in 10 μl RNase-free TE-buffer (10 mM Tris, 0.1 mM EDTA, pH 7.5). For reverse transcription (RT) and the subsequent PCR a pair of primers was designed from the conserved 5′-noncoding (5′-NC) region of the HCV genome (sense primer: 5′-ACGCAGAAAGCGTCTAGC-CATGGCGTTAGT-3′; antisense primer: 5′-TCCCGGGGCACT-CGCAAGCACCCTATCAGG-3′). Two different sets of primers were used for RT-PCR of the less conserved NS-5 region to ensure amplification of each genotype (sense primer-1: 5′-CTCCACAGT-CACTGAGAGCGACATCCGTAC-3′; anti-sense primer-1: 5′-AT-AGCCTCCGTGAAGGCTCTCAGGCTCGCC-3′; sense-primer-2: 5′-CTCAACTGTCACTGAACAGGACATCAGGGT-3′; anti-sense primer-2: 5′-ATAGCCTCCGTGAAGGCTCTCAGGGCT-CGT-3′). Reverse transcription was carried out with 2 μl of resuspended RNA (total volume: 20 μl) using the Gene Amp RNA-PCR-kit (Perkin Elmer, Langen, Germany) at 43°C for 30 minutes with the antisense primer (0.1 μM). cDNA was amplified in 50 μl of a solution containing 2.5 U Taq polymerase (Perkin Elmer Cetus, Norwalk, CT, USA), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 20 μM desoxynucleoside triphosphate by the addition of 0.1 μM sense primer. Denaturation was performed at 95°C for 30 seconds, primer annealing at 72°C (5′-NC) and 55°C (NS-5) for 40 seconds and extension at 72°C (5′-NC and NS-5) for 40 seconds per cycle in a PE 9600 thermocycler (Perkin Elmer Cetus). After a total of 50 cycles 10 μl of the amplification product of the 5′-NC and the NS-5 region of the HCV genome (−276 to −21 and +7936 to +8290, based on the numbering system of Choo et al [19, 20]) were analyzed on a 3% agarose gel stained with ethidium bromide. The remainder was purified with Centricon 100 (Amicon, Witten, Germany) and subsequently incubated with dideoxynucleotides (Dye Deoxy™ Terminators, Applied Biosystems, Weiterstadt, Germany), sense and antisense primers, respectively, for 30 cycles (96°C for 30 seconds, 50°C for 15 seconds, 60°C for 4 min) according to the manufacturer's instructions. The resulting single-stranded PCR products were again purified by phenol-chloroform and concentrated by ethanol precipitation. Sequencing of the (+)-strand and the (−)-strand was performed by an automat (Applied Biosystems 373A DNA Sequencer, Weiterstadt, Germany). All contamination prevention measures suggested by Kwok and Higuchi were strictly applied [21].

Phylogenetic analysis

Phylogenetic analysis of 5′-NC and NS-5 sequences obtained from HCV isolates of the investigated patients was performed using the program *Treecon for Windows* [22]. The matrix was calculated by use of the Kimura correction [23], whereas the tree was estimated by the neighbor-joining method [24]. Bootstrap resampling was utilized as a pseudo-empirical test on the reliability of the tree topology [25].

Statistical analysis

All results are expressed as mean ± SD. Differences between groups were analyzed by application of the Chi-square-test with Yates correction for non-parametric data.

Results

At the beginning of the study the mean age of the investigated hemodialysis patients was 56.7 years (range 19 to 79 years) and mean duration of hemodialysis treatment was 81 months (range 2 to 272 months). None of the anti-HCV negative hemodialysis patients tested positive for serum HCV-RNA, indicating a high specificity of the anti-HCV ELISA system in our study population. Serum ALT was elevated (> 23 U/liter) only in 5 of 16 anti-HCV positive hemodialysis patients, the mean ALT-level in this group was 19.6 ± 17.2 U/liter. Fifty-six patients with chronic hepatitis C and no evidence of renal disease served as anti-HCV and HCV-RNA positive controls. The mean age in this group was 47.9 years (range 22 to 75 years), serum ALT was elevated in each patient (mean 84.1 ± 47.7 U/liter; Table 1).

The transfusion history of the hemodialysis patients is shown in Table 2. Only 3 of 16 (18.8%) anti-HCV positive hemodialysis patients, but 81 of 208 (38.9%) anti-HCV negative patients had previously not received blood transfusions. The difference of anti-HCV prevalence in patients with and without previous blood transfusions failed to reach statistical significance (P = 0.1079). However, there was a significant correlation between the presence of anti-HCV and a history of more than 10 blood transfusions (P = 0.001). Six of 16 anti-HCV positive hemodialysis patients were previously kidney transplanted. All transplantations were performed before introduction of routine anti-HCV testing of organ donors in 1990 to 1991. Duration of hemodialysis treatment for less than 10 years was not related to the anti-HCV status. Six of 65 patients which were on maintenance hemodialysis for more than 10 years were anti-HCV positive (Table 3). The difference between anti-HCV positive (6 of 16) and negative (59 of 208) long-term hemodialysis patients was significant (P = 0.001).

To address the issue of patient-to-patient transmission in our hemodialysis unit we performed sequence and phylogenetic analyses of HCV isolates from all HCV-RNA positive hemodialysis patients. Direct sequencing of PCR products without intermediate cloning steps enables determination of the predominant viral sequence in a given patient. Nucleotide sequence analysis between position −276 and −21 (5′-noncoding region) of the HCV

Table 3. Anti-HCV antibodies in relation to the duration of hemodialysis treatment

Duration of hemodialysis treatment *years*	Anti-HCV negative patients (N = 208)	Anti-HCV positive patients (N = 16)	P value
< 2	69 (33.1%)	4 (25.0%)	NS
2–5	47 (22.6%)	4 (25.0%)	NS
6–10	33 (15.9%)	2 (12.5%)	NS
> 10	59 (28.4%)	6 (37.5%)	0.001

genoms isolated from 14 HCV-RNA positive hemodialysis patients revealed the presence of HCV genotype 1 in 11 of 14 (78.6%) and HCV genotype 2 in 3 of 14 (21.4%) cases (classification according to Simmonds et al [26]). The genotype distribution of 56 HCV-RNA positive patients with chronic hepatitis C but without evidence of renal disease from the same geographical area was: HCV-1 in 38 of 56 (67.9%); HCV-2 in 7 of 56 (12.5%); and HCV-3 in 11 of 56 (19.6%).

Figure 1 shows the phylogenetic tree for all HCV isolates (5′-noncoding region) from hemodialysis and control patients. Patients HD-KF and HD-SK were the only HCV-RNA positive hemodialysis patients who previously had not received blood transfusions and/or organ transplantation. The phylogenetic distribution of the HCV isolates reveals two small clusters of patients on maintenance hemodialysis (cluster 1, HD-KL and HD-SK; cluster 2, HD-BH and HD-JG). For further evaluation of the evolutionary distance between these HCV isolates, nucleotide sequence analyses in the less conserved NS-5 region (nucleotide positions +7936 to +8290) were performed. Sequence analyses of 9 (cluster 1) and 7 (cluster 2) closely related HCV isolates from non-hemodialysis patients served as controls.

In Figure 2 the phylogenetic tree of the NS-5 region for isolates of cluster 1 (Fig. 2A) and cluster 2 (Fig. 2B) is shown. The analysis provides strong evidence that isolates HD-KL and HD-SK are unrelated. However, phylogenetic analysis together with NS-5 sequences of other genotype HCV-2 isolates still places the sequences of HD-BH and HD-JG closely together. In our panel the evolutionary distance between the two sequences (9.6%) is smaller than that between most other NS-5 sequences of genotype HCV-2. However, the evolutionary distance between HCV isolate of patient HD-BH and an isolate of an unrelated control patient was even closer (5.1%; Fig. 2B).

Patient HD-BH and HD-JG had previously received 3 and 9 units of blood, respectively. Blood groups of the two patients were divergent excluding the possibility that both received blood from the same HCV-infected blood donor. Patient HD-JG was once kidney transplanted in 1985, the anti-HCV status of the donor is unknown. Both patients never shared the same hemodialysis machine; indeed, retrospective investigation revealed that hemodialysis was continuously performed in separate rooms. Detailed questionnaires gave no evidence for any known HCV transmission risk factor between the two patients.

Discussion

The prevalence of hepatitis C virus infection varies widely among dialysis units all over the world [6, 8–10, 12, 13, 27–32]. This variation is likely due to multiple factors, including the prevalence of HCV-RNA positive blood donors in different countries, the past transfusion practices and hygienic standards of hemodialysis units. As in non-hemodialyzed patients, a certain proportion of HCV infections might be due to yet unidentified transmission routes.

In the present study of 224 hemodialyzed patients we found a prevalence of anti-HCV antibodies (2nd generation ELISA) of 7.1%. Detection of anti-HCV antibodies was associated with persistent presence of HCV-RNA in most hemodialysis patients (14 of 16 cases; 87.5%). None of the anti-HCV negative patients tested positive for HCV-RNA by reverse transcription-polymerase chain reaction indicating that second generation ELISAs are sensitive and specific. Our RT-PCR assay is well validated and optimized, achieving an analytical sensitivity of 10 HCV-RNA molecules [16, 17]. Serum samples were obtained before hemodialysis treatment since heparin is a known inhibitor of reverse transcriptase and *Taq* DNA polymerase [33].

Low fidelity of HCV replication leading to a considerable degree of genome variability is attributed to a lack of proof reading activity of the RNA-dependent RNA polymerase encoded by the NS-5 region of HCV. Based on variations in nucleotide sequences of HCV isolates, several genotypes have been identified and subsequently classified into different typing schemes [26, 29, 34]. According to the classification proposed by Simmonds et al [26] we identified 78.6% of HCV isolates from hemodialysis patients as genotype HCV-1 and 21.4% as HCV-2. In the control group the prevalence rates for genotypes HCV-1, -2 and -3 were 67.9%, 12.5% and 19.6%, respectively. Several studies have recognized that European drug addicts are preferentially infected with genotype HCV-3 [35, 36]. Eleven control patients, but none of the hemodialysis patients, were infected with HCV-3. Intravenous drug abuse was identified as risk factor for HCV transmission only in controls but not in hemodialyzed patients. Six of 9 control patients with previous intravenous drug abuse were infected with genotype HCV-3.

Several studies have shown the value of molecular epidemiological techniques for identifying distant sources of infections, and for the epidemiological investigation of the current distribution and transmission of HCV in different populations [37–39]. Despite an overall mutation rate of about 2×10^{-3} mutations per nucleotide position per year [40], the rate of sequence change over time in the 5′-noncoding and NS-5 region is slow enough to preserve evidence of relatedness over a considerable period of time [37]. The much higher rate of sequence change, particularly in the envelope gene of HCV, may hinder attempts to link contacts and sources of infection [41, 42].

In the present study we phylogenetically analyzed all 14 isolates of HCV-RNA positive patients of our hemodialysis unit together with a control panel of 56 unrelated patients with chronic hepatitis C from the same geographical area. In the phylogenetic tree 10 of 14 HCV isolates from hemodialysis patients were randomly distributed within the control HCV isolates. Two small clusters (two patients each) were observed in the phylogenetic analysis of the 5′-noncoding region. Therefore the analysis of these hemodialysis patients together with (according to the 5′-noncoding region) closely related control isolates was extended to the less conserved NS-5 region. For cluster 1 (HD-KL and HD-SK) the phylogenetic analysis revealed no evidence for a single source of infection (Fig. 1). However, in cluster 2 both hemodialysis patients (HD-BH and HD-JG) were infected with similar variants (Fig. 2). Pair-wise comparisons of nucleotide sequences of HCV isolates

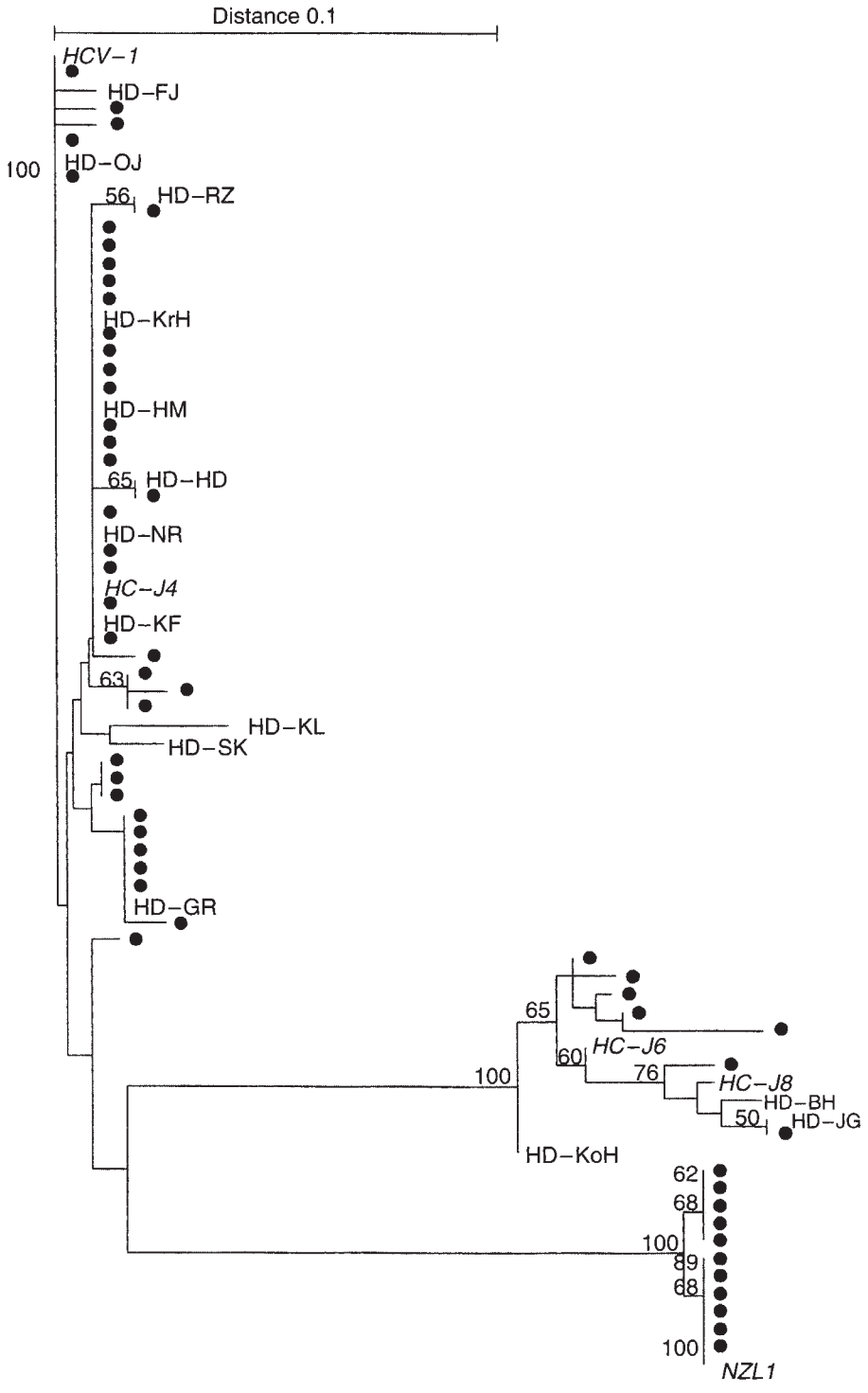

Fig. 1. *Phylogenetic analysis of the 5'-noncoding sequences obtained from HCV isolates of 14 hemodialysis patients and 56 controls.* The phylogenetic tree was constructed as described in the **Methods** section. Bootstrap resampling was utilized as a pseudo-empirical test on the reliability of the tree topology. The numbers at the forks show the number of occurrences of the respective group to the right out of 100 bootstrap samples. The distance scale represents 10% sequence dissimilarity. Hemodialysis patients are indicated by initials, controls as closed circles (●). Two small clusters of hemodialysis patients are observed: (1) HD-KL and HD-SK and (2) HD-BH and HD-JG. Reference sequences for respective genotypes: HCV-1 and HC-J4 (HCV-1a and b [20, 27]), HC-J6 (HCV-2a, [28]), HC-J8 (HCV-2b, [29]) and NZL1 (HCV-3, [30]). Discrimination of HCV subtypes 1a and 1b by phylogenetic analysis of the 5'-noncoding region is not reliable [31].

from patient HD-BH and HD-JG revealed a difference of 9.6% in the NS-5 region. A common blood donor for the two patients was ruled out, and both never shared the same room or dialysis equipment in our unit. Despite a limited number of closely related control isolates of genotype HCV-2 ($N = 7$), the phylogenetic analysis of the NS-5 sequences places one control isolate on a separate arm together with HD-BH (Fig. 2B). Thus, transmission of HCV in patients HD-BH and HD-JG might have occurred from different sources. According to an estimated mutation rate of 2×10^{-3} mutations per nucleotide position per year [40], and assuming that this rate is not accelerated in hemodialysis patients, a nucleotide difference of 9.6% between isolates indicates that the infection event occurred about 48 years ago. At the beginning of the study the two patients were 41 and 69 years old and were on

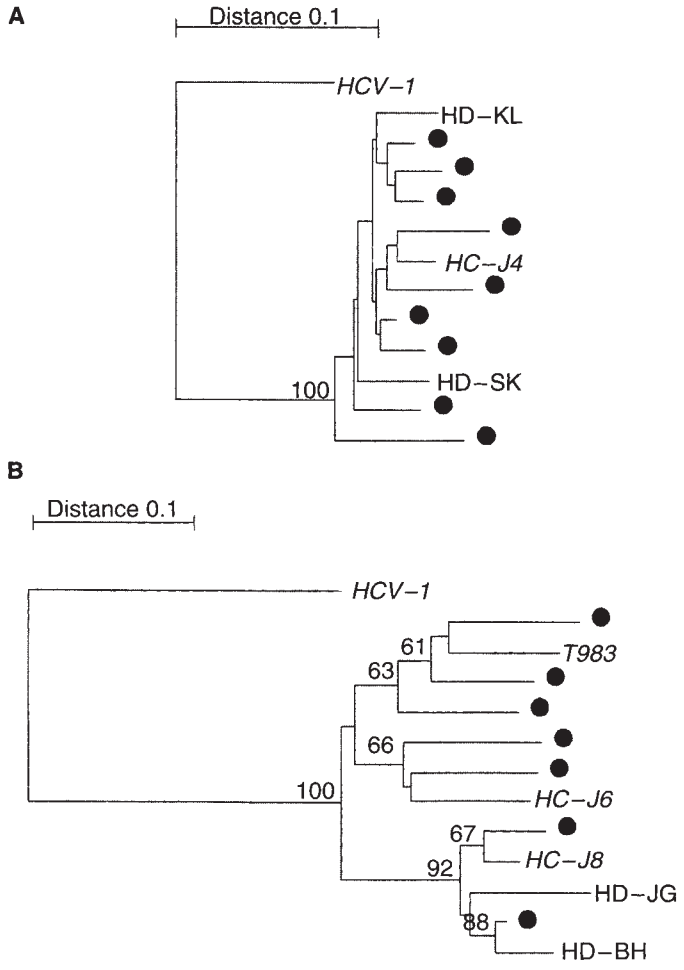

Fig. 2. *Phylogenetic analysis of NS-5 sequences obtained from HCV isolates of hemodialysis patients clustering in the phylogenetic tree of the 5'-noncoding region.* HCV-RNA was extracted from serum, reverse transcribed and the HCV-cDNA amplification product of the NS-5 region sequenced as described in the **Methods** section. The phylogenetic tree was constructed as described in the legend to Figure 1 using 9 and 7 closely related control isolates for cluster 1 (HD-KL and HD-SK) (**A**) and cluster 2 (HD-BH and HD-JG) (**B**), respectively. The numbers at the forks show the number of occurrences of the respective group to the right out of 100 bootstrap samples. The distance scale represents 10% sequence dissimilarity. Hemodialysis patients are indicated by initials, controls as closed circles (●). Reference sequences for genotypes in panel A: HCV-1 (HCV-1a [20]), HC-J4 (HCV-1b [27]) and in panel B: HCV-1 (HCV-1a [20]), HC-J6 (HCV-2a, [28]), HC-J8 (HCV-2b, [29]) and T983 (HCV-2c, [26]).

hemodialysis treatment for 31 and 20 months, respectively. Thus, despite a high homology of the respective HCV isolates, patient-to-patient transmission appears rather unlikely.

Nosocomial HCV infection of hemodialysis patients has recently been suspected in several studies investigating the relationship of anti-HCV with past blood transfusions and the duration of hemodialysis treatment. Similar to the present study a close relationship between anti-HCV and the duration of time for which the patient had been hemodialyzed was shown [12, 13, 32]. However, as shown in the present study such a relationship provides no proof for patient-to-patient infection. More convincing evidence for nosocomial infection comes from prospective studies on seroconversion for HCV in hemodialysis patients [11, 43].

Only few studies have yet applied molecular approaches to investigate the question of nosocomial infection between patients of a hemodialysis unit. Widell et al performed genotyping of hepatitis C virus isolates using genotype-specific primers for PCR amplification in a cluster of infected cases of a hemodialysis unit and found the presence of HCV-1b in 8 of 11 patients [44]. Further sequence analysis showed a homology between isolates of 98.5 to 100%. In this study a panel of unrelated control patients was not investigated. In a similar study by Allander et al sequence analyses of HCV isolates from 14 hemodialysis patients and 9 controls revealed the presence of the same viral strain in 5 of 14 hemodialysis patients [45]. Alignment of the viral cDNA sequences displayed several nucleotide substitutions between the 5 homologous HCV isolates from hemodialysis patients and the controls. Sampietro et al employed single strand conformation polymorphism (SSCP) analysis to evaluate the heterogeneity of HCV sequences [10]. The relative homogeneity of HCV variants in 28 patients on chronic hemodialysis supported the possibility of nosocomial transmission of HCV.

The present study applies phylogenetic analysis of nucleotide sequences in all HCV-RNA positive hemodialysis patients from one department and a panel of control isolates obtained from non-hemodialysis patients. This molecular epidemiological technique is the most powerful method to retrospectively investigate possible sources of HCV infection. Our data fail to provide convincing evidence for patient-to-patient transmission of HCV in our hemodialysis unit. Generally, we do not exclude this possibility of HCV transmission. However, in developed countries all equipment for hemodialysis including needles, tubes, and dialyzers have been disposable for at least 15 years. The staff of most hemodialysis units is usually well-educated and aware of the risk for transmission of blood-borne viruses. As yet there is no convincing evidence that adherence to strict infection preventive routines should not be sufficient to prevent spread of HCV.

From the data of the present study we like to draw attention to the possibility that HCV transmission might have occurred via transfusion of blood and blood products such as human serum albumin, fresh frozen plasma and immunoglobulins (including hepatitis B immune globulin). Recently, several studies have well documented the risk of HCV transmission from pooled plasma products [37, 46, 47]. Human serum albumin is used very frequently to control hypotension during hemodialysis. Insufficient virus inactivation procedures in very few preparations leading to a small remaining virus load may be able to cause HCV infection in the immunocompromised hemodialysis patients. Furthermore, in about 40% of non-dialyzing patients with chronic hepatitis C worldwide, no risk factor for HCV transmission can be established [3, 4]. We conclude that unless sequence and phylogenetic analysis of HCV isolates provide convincing evidence for patient-to-patient transmission, we should keep aware that other known and unknown transmission routes exist for HCV.

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References

1. VAN DER POEL CL, CUYPERS HT, REESINK HW: Hepatitis C virus six years on. *Lancet* 344:1475–1479, 1994
2. ZEUZEM S, ROTH WK, HERRMANN G: Virushepatitis C. *Z Gastroenterol* 33:117–132, 1995
3. HOLLINGER FB, LIN HJ: Community-acquired hepatitis C virus infection. *Gastroenterology* 102:1426–1429, 1992
4. BORTOLOTTI F, TAGGER A, CADROBBI P, CRIVELLARO C, PREGLIASCO F, RIBERO ML, ALBERTI A: Antibodies to hepatitis C virus in community-acquired acute non-A, non-B hepatitis. *J Hepatol* 12:176–180, 1991
5. ZELDIS JB, DEPNER TA, KURAMOTO IK, GISH RG, HOLLAND PV: The prevalence of hepatitis C virus antibodies among hemodialysis patients. *Ann Intern Med* 112:958–960, 1990
6. JEFFERS LJ, PEREZ GO, DE MEDINA MD, ORTIZ-INTERIAN CJ, SCHIFF ER, REDDY KR, JIMENEZ M, BOURGOIGNIE JJ, VAAMONDE CA, DUNCAN R, HOUGHTON M, CHOO G-L, KUO G: Hepatitis C infection in two urban hemodialysis units. *Kidney Int* 38:320–322, 1990
7. CHAN TM, LOK ASF, CHENG IKP, CHAN RT: Prevalence of hepatitis C virus infection in hemodialysis patients: A longitudinal study comparing the results of RNA and antibody assays. *Hepatology* 17:5–8, 1993
8. FABRIZI F, RAFFELE L, BACCHINI G, GUARNORI I, PONTORIERO G, ERBA G, LOCATELLI F: Antibodies to hepatitis C virus (HCV) and transaminase concentration in chronic hemodialysis patients: A study with second-generation assays. *Nephrol Dial Transplant* 8:744–747, 1993
9. KNUDSEN F, WANTZIN P, RASMUSSEN K, LADEFOGED SD, LOKKEGAARD N, RASMUSSEN LS, LASSEN A, KROGSGAARD K: Hepatitis C in dialysis patients: Relationship to blood transfusions, dialysis and liver disease. *Kidney Int* 43:1353–1356, 1993
10. SAMPIETRO M, BADALAMENTI S, SALVADORI S, CORBETTA N, GRAZIANI G, COMO G, FIORELLI G, PONTICELLI C: High prevalence of a rare hepatitis C virus in patients treated in the same hemodialysis unit: Evidence for nosocomial transmission of HCV. *Kidney Int* 47:911–917, 1995
11. JADOUL M, CORNU C, VAN YPERSELE DE STRIHOU C, UCL COLLABORATIVE GROUP: Incidence and risk factors for hepatitis C seroconversion in hemodialysis: A prospective study. *Kidney Int* 44:1322–1326, 1993
12. IRIE Y, HAYASHI H, YOKOZEKI K, KASHIMA T, OKUDA K: Hepatitis C infection unrelated to blood transfusion in hemodialysis patients. *J Hepatol* 20:557–559, 1994
13. OKUDA K, HAYASHI H, KOBAYASHI S, IRIE Y: Mode of hepatitis C infection not associated with blood transfusion among chronic hemodialysis patients. *J Hepatol* 23:28–31, 1995
14. DAVIS GL, LAU JYN, URDEA MS, NEUWALD PD, WILBER JC, LINDSAY K, PERRILLO RP, ALBRECHT J: Quantitative detection of hepatitis C virus RNA with a solid-phase signal amplification method: Definition of optimal conditions for specimen collection and clinical application in interferon-treated patients. *Hepatology* 19:1337–1341, 1994
15. BUKH J, PURCELL RH, MILLER, RH: Importance of primer selection for the detection of hepatitis C virus RNA with the polymerase chain reaction assay. *Proc Natl Acad Sci USA* 89:187–191, 1992
16. ZEUZEM S, RÜSTER B, ROTH WK: Clinical evaluation of a new polymerase chain reaction assay (Amplicor™ HCV) for detection of hepatitis C virus. *Z Gastroenterol* 32:342–347, 1994
17. RÜSTER B, ZEUZEM S, ROTH WK: Quantification of hepatitis C virus RNA by competitive reverse transcription and polymerase chain reaction using a modified HCV-RNA transcript. *Anal Biochem* 224: 597–600, 1995
18. CHOMCZYNSKI P, SACCHI N: Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156–159, 1987
19. CHOO QL, KUO G, WEINER AJ, OVERBY LR, BRADLY DW, HOUGHTON M: Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* 244:359–362, 1989
20. CHOO Q-L, RICHMAN KH, HAN JH, BERGER K, LEE C, DONG C, GALLEGOS C, COIT D, MEDINA-SELBY A, BARR PJ, WEINER AJ, BRADLEY DW, KUO G, HOUGHTON M: Genetic organization and diversity of the hepatitis C virus. *Proc Natl Acad Sci USA* 88:2451–2455, 1991
21. KWOK S, HIGUSHI R: Avoiding false positives with PCR. *Nature* 339:237–238, 1989
22. VAN DE PEER Y, DE WACHTER R: Treecon for Windows: A software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. *Comput Applic Biosci* 10:569–570, 1994
23. KIMURA M: A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* 16:111–120, 1980
24. SAITOU N, NEI M: The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4:406–425, 1987
25. FELSENSTEIN J: Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* 39:783–791, 1985
26. SIMMONDS P, HOLMES EC, CHA TA, CHAN SW, MCOMISH F, IRVINE B, BEALL E, YAP PL, KOLBERG J, URDEA MS: Classification of hepatitis C virus into six major genotypes and a series of subtypes by phylogenetic analysis of the NS-5 region. *J Gen Virol* 74:2391–2399, 1993
27. OKAMOTO H, KOJIMA M, OKADA S-I, YOSHIZAWA H, IIZUKA H, TANAKA T, MUCHMORE EE, PETERSON DA, ITO Y, MISHIRO S: Genetic drift of hepatitis C virus during an 8.2-year infection in a chimpanzee: Variability and stability. *Virology* 190:894–899, 1992
28. OKAMOTO H, OKADA S, SUGIYAMA Y, KURAI H, IIZUKA H, MACHIDA A, MIYAKAWA Y, MAYUMI M: Nucleotide sequence of the genomic RNA of hepatitis C virus isolated from a human carrier: Comparison with reported isolates for conserved and divergent regions. *J Gen Virol* 72:2697–2704, 1991
29. OKAMOTO H, KURAI K, OKADA S-I, YAMAMOTO K, LIZUKA H, TANAKA T, FUKUDA S, TSUDA F, MISHIRO S: Full-length sequence of a hepatitis C virus genome having poor homology to reported isolates: Comparative study of four distinct genotypes. *Virology* 188:331–341, 1992
30. SAKAMOTO M, AKAHANE Y, TSUDA F, TANAKA T, WOODFIELD DG, OKAMOTO H: Entire nucleotide sequence and characterization of a hepatitis C virus of genotype V/3a. *J Gen Virol* 75:1761–1768, 1994
31. ZEUZEM S, RÜSTER B, LEE J-H, STRIPF T, ROTH WK: Evaluation of a reverse hybridization assay for genotyping of hepatitis C virus. *J Hepatol* (in press)
32. HARDY NM, SANDRONI S, DANIELSON S, WILSON WJ: Antibody to hepatitis C virus increases with time on hemodialysis. *Clin Nephrol* 38:44–48, 1992
33. IZRAELI S, PFLEIDERER C, LION T: Detection of gene expression by PCR amplification of RNA derived from frozen heparinized whole blood. *Nucl Acids Res* 19:6051, 1991
34. MORI S, KATO N, YAGYU A, TANAKA T, IKEDA Y, PETCHCLAI B, CHIEWSILP P, KURIMURA T, SHIMOTOHNO K: A new type of hepatitis C virus in patients in Thailand. *Biochem Biophys Res Commun* 183:334–342, 1992
35. POL S, THIERS V, NOUSBAUM J-B, LEGENDRE C, BERTHELOT P, KREIS H, BRECHOT C: The changing relative prevalence of hepatitis C virus genotypes: Evidence in hemodialyzed patients and kidney recipients. *Gastroenterology* 108:581–583, 1995
36. SILINI E, BONO F, CERINO A, CIVIDINI A, MACCABRUNI A, BABARINI G, MONDELLI MU: Molecular epidemiology of HCV infection in drug addicts. (abstract) *J Hepatol* 21:S33, 1994
37. POWER JP, LAWLOR E, DAVIDSON F, HOLMES EC, YAP PL, SIMMONDS P: Molecular epidemiology of an outbreak of infection with hepatitis C virus in recipients of anti-D immunoglobulin. *Lancet* 345:1211–1213, 1995
38. CHAYAMA K, KOBAYASHI M, TSUBOTA A, KOIDA I, ARASE Y, SAITOH S, IKEDA K, KUMADA H: Molecular analysis of intraspousal transmission of hepatitis C virus. *J Hepatol* 22:431–439, 1995
39. ALLANDER T, GRUBER A, NAGHAVI M, BEYENE A, SÖDERSTRÖM T, BJÖRKHOLM M, GRILLNER L, PERSSON MAA: Frequent patient-to-patient transmission of hepatitis C virus in a haematology ward. *Lancet* 345:603–607, 1995

40. OGATA N, ALTER HJ, MILLER RH, PURCELL RH: Nucleotide sequence and mutation rate of the H strain of hepatitis C virus. *Proc Natl Acad Sci USA* 88:3392–3396, 1991
41. OU CY, CIESIELSKI CA, MYERS G, BANDEA CI, LUO C-C, KORBER TM, MULLINS JI, SCHOCHETMAN G, BERKELMAN RL, ECONOMOU AN, WITTE JJ, FURMAN LJ, SATTEN GA, MACINNES KA, CURRAN JW, JAFFE HW, LABORATORY INVESTIGATION GROUP, EPIDEMIOLOGIC INVESTIGATION GROUP: Molecular epidemiology of HIV transmission in a dental practice. *Science* 256:1165–1171, 1992
42. HOLMES EC, LEIGH BROWN AJ, SIMMONDS P: Sequence data as evidence. *Nature* 364:766, 1993
43. SIMON N, COUROUCÉ A-M, LEMARREC N, TRÉPO C, DUCAMP S: A twelve year natural history of hepatitis C virus infection in hemodialyzed patients. *Kidney Int* 46:504–511, 1994
44. WIDELL A, SHEV S, MANSSON S, ZHANG Y-Y, FOBERG U, NORKRANS G, FRYDÉN A, WEILAND O, KURKUS J, NORDENFELT E: Genotyping of hepatitis C virus isolates by a modified polymerase chain reaction assay using type specific primers: Epidemiological applications. *J Med Virol* 44:272–279, 1994
45. ALLANDER T, MEDIN C, JACOBSON SH, GRILLNER L, PERSSON MAA: Hepatitis C transmission in a hemodialysis unit: Molecular evidence for spread of virus among patients not sharing equipment. *J Med Virol* 43:415–419, 1994
46. YU MW, MASON BL, GUO ZP, TANKERSLEY DL, NEDJAR S, MITCHELL FD, BISWAS RM: Hepatitis C transmission associated with intravenous immunoglobulins. *Lancet* 345:1173–1174, 1995
47. NÜBLING CM, WILLKOMMEN H, LÖWER J: Hepatitis C transmission associated with intravenous immunoglobulins. *Lancet* 345:1174, 1995